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Metabolically based resistance to the herbicide propanil in *Echinochloa* species

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Propanil is an acylanilide herbicide introduced in the early 1960s to control dicotyledonous weeds and grasses, including *Echinochloa* species in cultivated rice. Since then, propanil has been used extensively in rice production in the United States and in several other countries. Propanil is an inhibitor of photosystem II, but rice is tolerant to propanil because of the presence of a high level of aryl acylamidase that catalytically degrades the compound to nonphytotoxic products, i.e., 3,4-dichloroaniline and propionic acid. About 10 yr ago, biotypes of barnyardgrass and junglerice were discovered to be resistant to propanil. The resistance mechanism of these two biotypes has been shown to be elevated levels of aryl acylamidase activity. Various strategies to combat propanil resistance and to more fully understand the biochemistry involved in this resistance have been investigated. These include studies on the interactions of herbicides and other chemicals with propanil, rotation of rice with other crops (consequently the use of other herbicide modes of action), and use of alternative herbicides in rice. Certain compounds, including some organophosphate insecticides, are potent inhibitors of aryl acylamidase, which can act as synergists with propanil to increase phytotoxicity. Another compound that lacks insecticidal or herbicidal activity, PPG-124, has been commercialized as a herbicide synergist for propanil. These chemical and biochemical interactions and other factors involved in propanil-resistant *Echinochloa* weeds are presented and discussed.

Nomenclature: 3,4-Dichloroaniline; PPG-124; propanil; barnyardgrass, *Echinochloa crus-galli* (L.) Beauv. ECHCG; junglerice, *Echinochloa colona* (L.) Link ECHCO; rice, *Oryza sativa* L.

Key words: Aryl acylamidase, chlorophyll fluorescence, herbicide interaction, herbicide metabolism, photosystem II, weed resistance.

There are two *Echinochloa* grass species, barnyardgrass (BYG) and junglerice (JR) that are especially troublesome weeds. BYG has had the distinction of being the world's worst weed in rice (Holm et al. 1977). This weed can cause up to 75% reduction in rice grain yield (Carey 1994), and a BYG density as low as 1 plant m^{-2} can reduce grain yield (Stauber et al. 1991). Season-long interference from BYG at a density of 57 plants m^{-2} reduced rice yield 50% (Smith 1988). Propanil, an acylanilide herbicide synthesized by Rohm and Haas in 1957 (Eberlein 1990), was introduced into cultivated rice in the United States in 1962 for broad-spectrum, postemergence control of dicotyledonous and monocotyledonous weeds including *Echinochloa* spp. (Smith 1961). Propanil was later labeled for use in wheat (*Triticum aestivum* L.) for foxtail (*Setaria* spp.) and broadleaf weed control (Eberlein 1990). Propanil has been used extensively in rice production in the United States in all rice-producing states (Arkansas, California, Florida, Louisiana, Missouri, Mssissippi, and Texas) and in several other countries but is no longer labeled for use in California. The mode of action of propanil is inhibition of photosystem II (PSII), but rice is tolerant to propanil because of the presence of high levels of the enzyme aryl acylamidase, which catalytically degrades the molecule to nonphytotoxic compounds, i.e., 3,4,-dichloroaniline and propionic acid (Frear and Still 1968) (Figure 1).

Development and Distribution of Propanil Resistance in *Echinochloa* spp.

About 10 yr ago, various populations of BYG and JR were found to exhibit resistance to propanil applied at label-recommended use rates of 3.6 to 5.6 kg ai ha^{-1}. In Arkansas, BYG resistance was reported in several geographical areas (Carey et al. 1992; Smith and Baltazar 1993). Likewise, in other rice-producing countries, certain BYG and JR biotypes began to exhibit resistance to propanil (Fischer et al. 1993; Garro et al. 1991).

Propanil-resistant BYG (R-BYG) was reported in Poinsett County, AK, in 1990 (Baltazar and Smith 1994). Propanil resistance was then confirmed in 115 (16 counties) of 138 Arkansas BYG seed sources collected in 1991 and 1992 (Carey et al. 1995b). The incidence of R-BYG populations appeared to be greatest along the rice-growing area of eastern Arkansas (Figure 2). In addition, propanil resistance was verified in the southwestern Red River valley (Lafayette County) and western Arkansas River valley (Logan County) regions. Through a producer survey, it was determined that propanil resistance was most often found in fields where rice was grown for many years (Carey et al. 1995b). Of the locations surveyed, 49% had been planted with rice for more than 20 yr, 20% for 15 to 20 yr, 27% for 5 to 15 yr, and 4% for less than 5 yr. The widespread distribution of R-BYG in Arkansas, as well as the correlation between crop

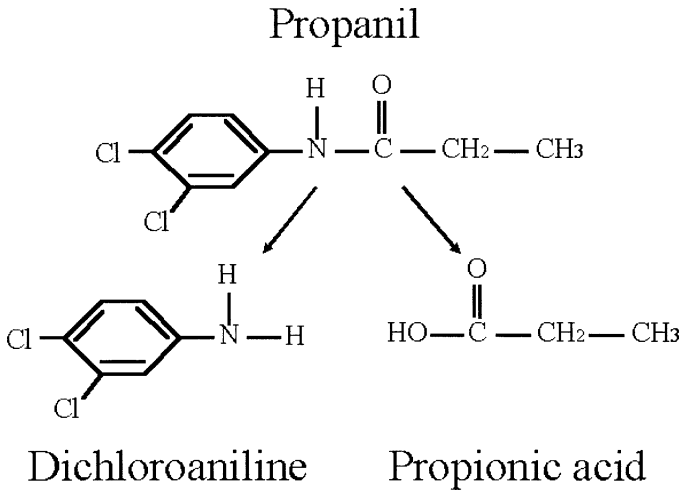

FIGURE 1. Chemical structure depicting the detoxification pathway of propanil by aryl acylamidase in plants, leading to the formation of 3,4-dichloroaniline and propionic acid.

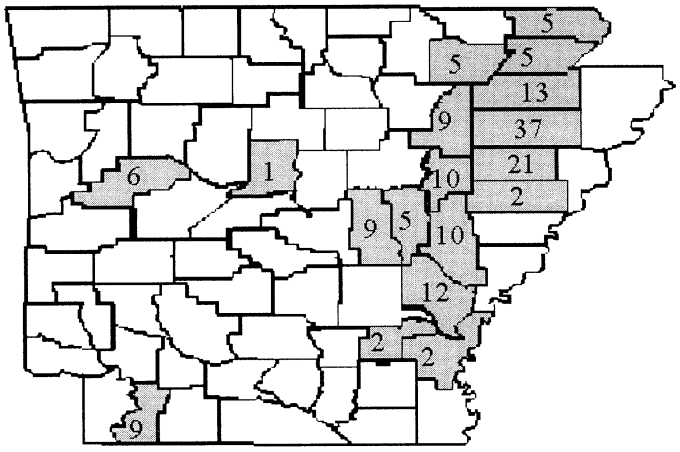

FIGURE 2. Distribution of propanil-resistant barnyardgrass in rice-producing counties of Arkansas. (Adapted from Norsworthy et al. 1998.)

rotation and propanil resistance, led researchers to hypothesize that resistance was developing independently rather than from a single point source (Carey et al. 1995b). This hypothesis was later confirmed using molecular techniques that determined that resistance developed from at least two genetically distinct biotypes (Rutledge et al. 2000).

In addition to the Arkansas seed samples, two samples were obtained from Louisiana, one from Missouri, and 21 from Texas for comparison in 1992. The samples from Louisiana and Missouri were susceptible to propanil, even though R-BYG had been verified in Louisiana. Over 50% of the Texas samples (collected from rice-producing areas near East Bernard, TX) were determined to be propanil resistant. However, 11 of these samples were later identified as JR. Of the 11 JR samples, four were susceptible and seven were resistant to propanil. In addition to Texas, propanil-resistant JR (R-JR) has been verified in rice-producing areas of Columbia (Fischer et al. 1993), Costa Rica (Garro et al. 1991), Central America (El Salvador, Guatemala, Nicaragua, and Panama) (Valverde et al. 2000), Venezuela (Ortiz et al. 1999), and Mexico (Villa-Casarez 1998). Propanil R-BYG now has been reported also in Greece (Giannopolitis and Vassiliou 1989), Sri Lanka (Marambe et al. 1997), Thailand (Maneechote and Krasaesindhu 1999), and Italy (Busi et al. 2003).

The 10 BYG accessions from Texas included six susceptible and four propanil-resistant samples (Carey et al. 1995b). Of the six states in the United States that apply propanil to rice (Arkansas, Florida, Louisiana, Mississippi, Missouri, and Texas), all but Florida have verified populations of R-BYG (Carey et al. 1995b). Recently in California, one accession of early watergrass *(Echinochloa oryzoides*) was found to be resistant to thiobencarb, and two accessions of late watergrass (*Echinochloa phyllopogon*) were resistant to molinate, thiobencarb, fenoxaprop-ethyl, and bispyribac-sodium (Fischer et al. 2000). None were resistant to propanil. In Arkansas, new herbicide-resistant accessions of BYG, which are resistant to propanil and quinclorac (Lovelace et al. 2002) or to quinclorac alone (M. L. Lovelace, personal communication), were discovered recently. Quinclorac-resistant BYG developed in Spain only 5 yr after introduction of this herbicide (De Prado et al. 1997). Recently, quinclorac-resistant BYG has been reported in Brazil (Valverde and Itoh 2001). These examples of resistance, and other weeds associated with rice production that have developed resistance to herbicides, are summarized elsewhere (Valverde and Itoh 2001).

Initial Detection and Evaluation of Propanil Resistance in BYG

Shortly after the confirmation of R-BYG in Arkansas (Smith and Baltazar 1993), a screening evaluation was developed (Carey et al. 1995b). Seeds from plants thought to be propanil resistant were harvested each fall by rice producers and sent to the University of Arkansas at Fayetteville, AR. These seeds were planted, and seedlings were grown to the two- to three-leaf stage in a greenhouse or growth chamber. They were then treated with 4.5 kg ha^{-1} propanil (recommended label rate), followed by visual injury or control ratings, three times during a 3-wk period. This whole procedure required approximately 10 mo, beginning with propanil failure in the field and ending with laboratory testing for resistance. Carey et al. (1995b) reported that between 1991 and 1992, 154 samples were screened for resistance using this technique, of which 138 were determined to be resistant. Since then, through 1996, propanil-resistant biotypes have been identified at 163 locations in 18 of the 38 rice-producing counties in Arkansas (Norsworthy et al. 1998) (Figure 2). Because of the lengthy procedure for screening plants for resistance, a rapid assay based on chlorophyll fluorescence was developed that allowed determination of R-BYG or propanil-susceptible BYG (S-BYG) seedlings (Norsworthy et al. 1998).

Quantification of Propanil Resistance in *Echinochloa* Species

BYG grown from seed samples collected throughout Arkansas responded differently to increasing rates of propanil (Carey et al. 1995b). S-BYG was controlled more than 90% by 6.7 kg ha^{-1} propanil and higher rates, whereas 20 kg ha^{-1} propanil was needed to control slightly resistant BYG more than 90%. An illustration of greenhouse-grown seed-

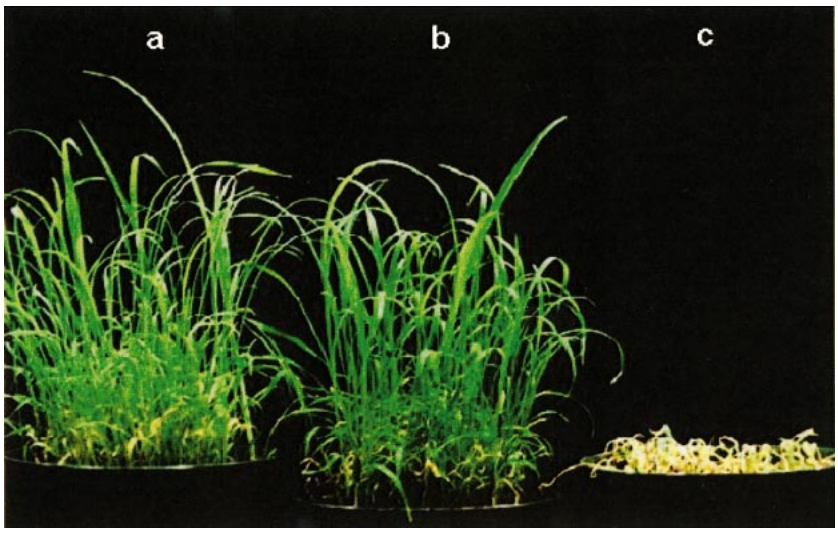

FIGURE 3. Visual illustration of the resistance and susceptibility of barnyardgrass to propanil applied at the three- to four-leaf growth stage: (a) untreated control, (b) resistant biotype treated with 20 kg ai ha^{-1} propanil, and (c) susceptible biotype treated with the recommended label rate of propanil, 4.5 kg ha^{-1}. Photo courtesy of N. Burgos.

lings of S-BYG that were completely controlled with 4.5 kg ha^{-1} vs. R-BYG treated with 20 kg ha^{-1} that exhibited no injury is presented in Figure 3. I_{50} values for each resistant category were < 6.7, 14, 20, and 39 for susceptible and slightly, moderately, and highly resistant biotype categories, respectively. A positive correlation was found between the propanil resistance category and the I_{50} rate of propanil. Differences in I_{50} values indicate that there are several levels of resistance present in R-BYG, as also was found in R-JR in Columbia (Fischer et al. 1993) and R-BYG in Greece (Giannopolitis and Vassiliou 1989). Because differing methodology was used for the calculation of resistance factors in R-BYG and R-JR, it is not possible to make valid quantitative comparison among biotypes. However, the above examples (and others not shown) clearly demonstrate that resistance to propanil exists at various levels in BYG and JR biotypes, imposing a detrimental economic impact on rice production in several countries.

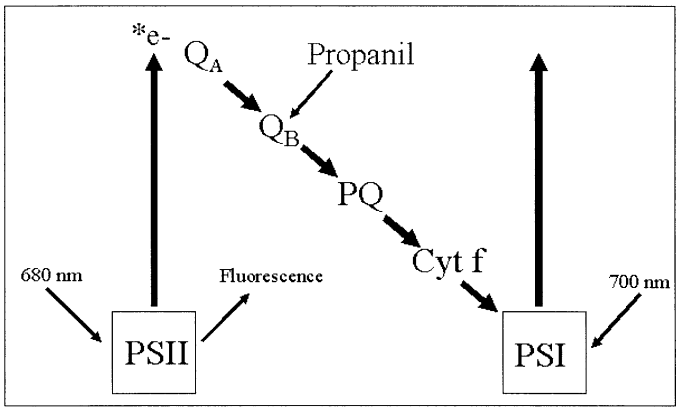

FIGURE 4. Schematic illustration of the inhibition site of propanil in photosystem II (PSII), depicting light energy dissipating as fluorescence when propanil blocks the electron flow through PSII.

Methods of Detecting Propanil Resistance in *Echinochloa* Species

Generally, there are two classes of detection methods, i.e., qualitative and quantitative, for determining resistance of weed biotypes to propanil. As pointed out previously, the most empirical method of assessing propanil resistance in BYG or JR is by general visual observations. For example, certain populations of BYG are not controlled with the normal recommended rates of propanil. Such qualitative observations require methods that are more quantitative to verify, confirm, and characterize the degree of herbicide resistance.

The initial quantitative screening procedure for determining propanil resistance in BYG required approximately 10 mo; therefore, a more rapid bioassay was needed and developed (Norsworthy et al. 1998). PSII inhibitors, such as propanil, block electron flow through PSII by binding to the QB site at the D1 protein, causing light energy to be dissipated as fluorescence (Harris and Camlin 1988) (Figure 4). Fluorescence measurements can be used to monitor the movement of PSII herbicides in vivo, examine for cross-resistance to herbicides, study the metabolic detoxification of PSII herbicides, and screen compounds for possible inhibition of photosynthetic electron transport (Gleiter and Renger 1993; Harris and Camlin 1988). Because propanil inhibits electron transport and the degree of inhibition varies between R-and S-BYG biotypes, a fluorescence assay can be used to rapidly distinguish biotypes. Because of the fact that various conditions can alter the results obtained in fluorescence measurements, several parameters (plant age, exposure time, propanil concentration, temperature effects, and shipment effects) were optimized for use in a standard assay.

A brief overview of the development of this bioassay (Norsworthy et al. 1998) follows. Chlorophyll fluorescence data from 13- to 41-d-old excised R- and S-BYG leaf tissue, exposed to 100 μM propanil for 2 h, exhibited no differences in inhibition of electron transport (photosynthesis in-

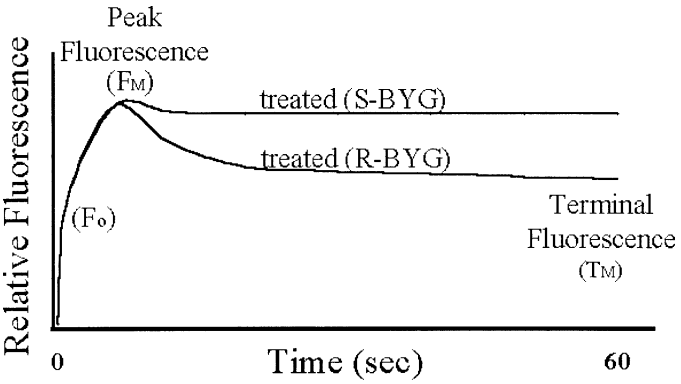

FIGURE 5. Illustration of typical fluorescence transient curves for propanil-resistant and -susceptible barnyardgrass treated with propanil and followed by a time period for possible metabolism of propanil. (Adapted from Norsworthy et al. 1998.)

hibition). However, if incubated in water in the dark for 22 h after an initial 2-h treatment, metabolism in R-BYG was sufficient to reduce the levels of absorbed propanil, allowing the two biotypes to be distinguished easily by the chlorophyll fluorescence assay. A herbicide dose–response curve showed the greatest difference in photosynthesis inhibition between biotypes at about 100 μM propanil, and both biotypes were inhibited > 95% when treated with 400 μM propanil. Inhibition of photosynthesis in both biotypes was greatest at 35 C compared with 20, 25, and 30 C. Regardless of plant age (plants larger than the four-leaf stage), biotypes could be separated using the chlorophyll fluorescence bioassay (Figure 5). Fluorescence data from harvested tissue stored in moist plastic bags at 23 C (to simulate shipment) showed that biotypes could be differentiated up to 4 d after harvest. Thus, samples could be harvested from the field soon after propanil failure, and resistance or susceptibility to propanil could be determined after only a few days. This technique greatly reduced the time, space, and labor required to determine propanil resistance in BYG.

Optimizing the evaluated parameters proved the use of fluorescence as an efficient technique for detection and confirmation of R-BYG. The technique required that aboveground tissue from 13- to 41-d-old BYG be tested within 4 d after harvest. Leaf segments must be floated on 100 μM propanil for 2 h and then placed in deionized water in the dark for 22 h at 20 to 30 C. Chlorophyll fluorescence measurements are then used for determination of resistance or susceptibility of BYG to propanil. A low level of photosynthesis inhibition in R-BYG indicated propanil metabolism.

Other detection methods have been developed and used elsewhere. In JR, germination of seeds in solutions of propanil at various concentrations has also been used as a rapid method for detecting propanil resistance (Kim et al. 2000). Leah et al. (1995) applied a propanil droplet to the adaxial leaf surface of JR and based resistance on the area of necrosis forming around the droplet.

Propanil injury to rice as well as increased BYG control often occur at temperatures of 35 C or higher. This may be attributed to greater propanil absorption and reduced aryl acylamidase activity, subsequently leading to reduced propanil metabolism at higher temperatures. For instance, Hoagland (1978) reported that in vitro aryl acylamidase activity from red rice decreases at exposure temperatures above 35 C. Similarly, wild rice species (*Oryza* spp.) have greater aryl acylamidase activity when grown at 20 to 25 C than at 30 C (Jun and Matsunaka 1990). At 32 C, propanil absorption is rapid, allowing more herbicide to reach the site of action than at lower temperatures (Hodgson 1971). In R-BYG, photosynthesis was not inhibited at 20, 25, and 30 C when floated on 100 μM propanil for 2 h followed by a 22-h dark recovery period, whereas inhibition in R-BYG did occur at 35 C, making the susceptible and resistant biotype difficult to distinguish using the chlorophyll fluorescence bioassay (Norsworthy et al. 1998).

Determination of the Propanil Resistance Mechanism

Uptake and Translocation in R-BYG

Laboratory studies were conducted using R- and S-BYG biotypes and ^{14}C-radiolabeled propanil to determine whether differential absorption–translocation of propanil was responsible for resistance (Carey et al. 1995a). Propanil absorption increased through a 48-h test period, with only 6% of the applied ^{14}C-propanil absorbed into BYG after 48 h. When ^{14}C-propanil was applied in an emulsion of formulated propanil and water to plants previously sprayed with unlabeled propanil, to simulate a field situation, absorption in R-BYG, S-BYG, and rice was similar, indicating that propanil resistance in BYG was not due to differential propanil absorption. Leah et al. (1995) also reported similar uptake of propanil by resistant and susceptible biotypes of JR at various growth stages and that uptake decreased with plant age for both biotypes, which inherently contributed to the difficulty of controlling larger plants.

In propanil translocation studies in R- and S-BYG, the majority (91%) of the absorbed ^{14}C-propanil remained in the treated leaf, with the remaining 3.2, 0.8, and 5.0% recovered in the foliar tissue above the treated leaf, the foliar tissue below the treated leaf, and root tissue, respectively (Carey et al. 1995a). Similarly, propanil uptake does not differ between propanil-susceptible JR (S-JR) and R-JR biotypes (Leah et al. 1995). These findings are consistent with those of others who found propanil translocation patterns for BYG and rice to be similar, even though biotypic morphological differences exist, leading to the conclusion that other factors are responsible for the differential herbicide selectivity between the weed and the crop (Still and Kuzirian 1967; Yih et al. 1968a).

Tests for Cross-resistance to Other PSII Inhibitors in R-BYG

In greenhouse studies, rice and both BYG biotypes were controlled 100% by atrazine, diuron, fluometuron, and linuron 10 d after emergence (Carey et al. 1995a). Earlier observations indicated no differential response between the BYG biotypes to any of the photosynthesis-inhibiting herbicides, indicating a lack of cross-resistance in R-BYG. Likewise, chlorophyll fluorescence data indicated that photosynthesis was inhibited similarly by atrazine (50 μM) in R- and S-BYG. The R-BYG biotype was not cross-resistant to other herbicides that inhibit PSII (atrazine, diuron, fluometuron, or linuron), based on equal mortality rates when treated with the recommended rates of these herbicides. Thus, pro-

panil resistance in the R-BYG biotype was not due to modification of the herbicidal site of action as it is in triazine-resistant biotypes.

Evaluation of Propanil Site of Action in R-BYG

Photosynthetic electron transport through PSII, measured by chlorophyll fluorescence, was inhibited in S-BYG when leaf disks were incubated in 50 μM propanil. Inhibition was > 75% by 1.5 h, and increased to > 90% after 5 h incubation, indicating that propanil interfered with photosynthetic electron transport in S-BYG. Fluorescence transients of propanil-treated tissues were similar to those of other PSII inhibitors (Bose et al. 1984; Gohbara et al. 1988). Photosynthesis in R-BYG was inhibited 50% when leaf disks were incubated in 50 μM propanil for 1.5 h. Inhibition decreased as incubation time in water after treatment increased, indicating that R-BYG was initially affected by propanil but then recovered. If the resistance mechanism in R-BYG was due to a change in the photosynthetic electron transport chain as in triazine resistance, there would have been no initial effect (Ahrens et al. 1981; Gohbara et al. 1988).

The insecticide carbaryl, which competitively inhibits the aryl acylamidase responsible for propanil metabolism in rice (Frear and Still 1968), was used with propanil in preliminary tests to block possible propanil metabolism in R-BYG (Carey et al. 1994; 1995a). When leaf disks of R- and S-BYG were incubated in 30 mM carbaryl, photosynthesis was not affected. However, when leaf disks of both biotypes were incubated for 5 h in a mixture of propanil and carbaryl, photosynthesis was completely inhibited. This carbaryl effect, combined with the apparent recovery phase of the propanil-resistant biotype, strongly suggested that the resistance mechanism in R-BYG was not due to molecular binding changes in the photosystem electron transport chain but that propanil was metabolized by the resistant biotype (Carey et al. 1994). These results are similar to data showing that carbaryl inhibited the resistance mechanism, resulting in greater injury by propanil in R-JR plants (Leah et al. 1995).

Tests to Evaluate Metabolism as a Resistance Mechanism in R-BYG

R-BYG populations, previously verified in Arkansas rice fields and greenhouse tests, were examined in the laboratory to ascertain whether the resistance mechanism in this weed biotype was by herbicide metabolism. R-BYG was controlled > 95% in the greenhouse when carbaryl was applied 2 d before propanil (Figure 6).

S-BYG was injured 55 to 80% by 4.5 kg ha^{-1} propanil over a period of 3 to 21 d after treatment (DAT) (Figure 6A). R-BYG injury ranged from about 28 to 40% in the same time period, whereas rice injury was < 10% from 3 to 7 DAT, with recovery at 14 DAT (Figure 6A). Carbaryl (1.1 kg ha^{-1}) applied alone gave no observable phytotoxic effects on rice or either BYG biotype. Propanil applied 2 d after carbaryl (1.1 kg ha^{-1}) increased rice injury to 30% at 21 DAT (Figure 6B) compared with 0% injury when propanil was applied alone (Figure 6A). R- and S-BYG were injured equally at all rating dates, and mortality was 98% at 21 DAT. The increase in injury of R-BYG when carbaryl was applied before propanil indicated that carbaryl overcame the resistance mechanism in R-BYG. These data indicated

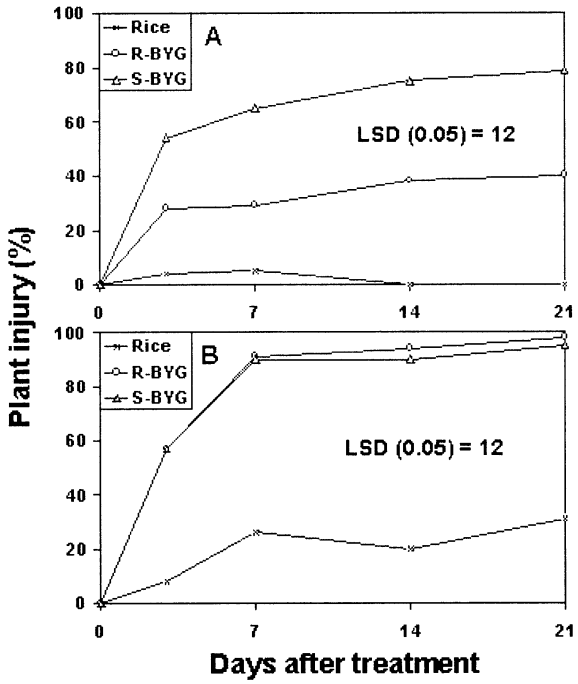

FIGURE 6. Responses of propanil-resistant (●) and -susceptible (▲) barnyardgrass and rice (■) at the two- to three-leaf stage to (A) propanil (4.5 kg ha^{-1}) applied in the greenhouse with no prior treatment and (B) propanil (4.5 kg ha^{-1}) applied in the greenhouse 2 d after carbaryl (1.1 kg ha^{-1}) treatment. (Adapted from Carey et al. 1997.)

that propanil may be metabolized in the R-BYG in a manner similar to that in rice.

Carey et al. (1995a) reported a lack of differential absorption of propanil among R-BYG, S-BYG, and rice. Leah et al. (1995) reported similar uptake of propanil by resistant and susceptible biotypes of JR at various growth stages. Uptake did decrease with plant age for both biotypes. Aryl acylamidase activity in R-JR was higher than that in the susceptible biotype at all growth stages. Fischer et al. (1996) reported that plant extracts from two- to three-leaf R-JR had higher levels of propanil metabolism than the susceptible biotype. As JR matured, the difference in degradation decreased. At 30 d after emergence, there was no difference in propanil degradation rate. Hence, the selectivity exhibited by plants of different ages is a function of aryl acylamidase activity and is also dependent on the absorption of propanil into leaf tissue.

Uptake of ^{14}C-propanil from solution by excised leaves was similar in rice, S-BYG, and R-BYG, and total recovery of ^{14}C averaged 90% (Carey et al. 1995a). A higher proportion of ^{14}C-propanil absorbed by leaves was extractable with methanol in S-BYG than in rice or R-BYG. Likewise, a higher degree of nonextractable radioactivity was present in rice and R-BYG. These results suggest a more extensive metabolism and incorporation of the radiolabeled products into lignin in tissues of the latter two seedlings.

Laboratory studies with ^{14}C-radiolabeled propanil indicated that the herbicide was hydrolyzed in R-BYG and rice to form 3,4-dichloroaniline and propionic acid, but no detectable hydrolysis occurred in S-BYG (Carey et al. 1997). Propanil and dichloroaniline (DCA) standards were separated using an acetone–benzene solvent system with R_f values of 0.35 and 0.5 for propanil and DCA, respectively (Figure

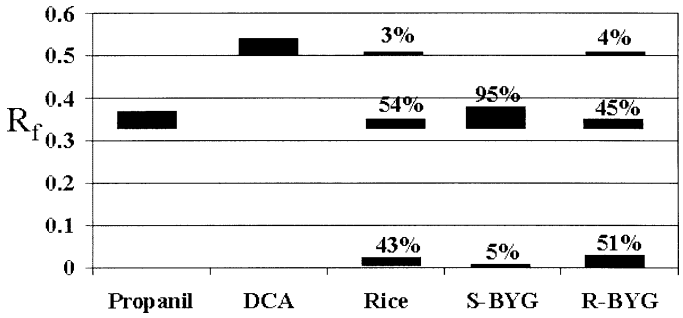

FIGURE 7. Diagram of a developed silica gel thin-layer chromatography plate showing the relative location, R_f value, and relative concentration of extractable ^{14}C after administering ^{14}C-propanil to barnyardgrass leaves. Abbreviations: R-BYG, propanil-resistant barnyardgrass; S-BYG, propanil-susceptible barnyardgrass. Solvent system: acetone + benzene (1 + 10, v/v). (Adapted from Carey et al. 1997.)

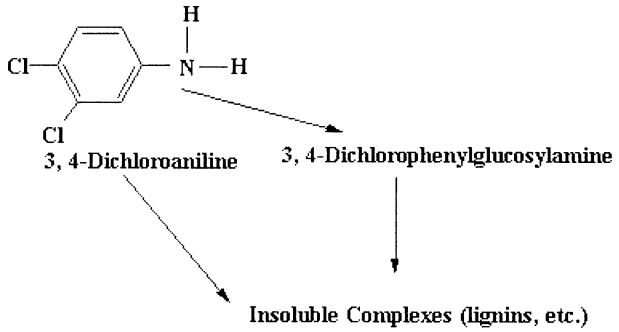

FIGURE 8. Degradation scheme of 3,4-dichloroaniline in plants.

7). In the extract of rice (four-leaf growth stage) incubated for 16 h, the majority of recovered ^{14}C was propanil, as identified by comparison with an authentic standard. However, 3% of recovered ^{14}C was DCA, which indicated that propanil was hydrolyzed in rice to form DCA. In S-BYG, no ^{14}C-DCA was recovered, indicating a lack of metabolism. These results are similar to other reports (Frear and Still 1968; Still and Kuzirian 1967; Yih et al. 1968a) that identified the production of DCA from propanil as the selectivity mechanism for BYG control in rice. In R-BYG, both ^{14}C-propanil and ^{14}C-DCA were recovered, indicating a similar metabolic reaction in R-BYG and in rice (Figure 7). Therefore, the resistance mechanism in R-BYG appeared to be metabolic degradation of the propanil molecule.

A large portion of radioactivity remained at the origin in the rice and R-BYG lanes when TLC plates were developed in the acetone–benzene solvent system. These highly polar metabolites have also been reported by Frear and Still (1968), Yih et al. (1968a, 1968b), and Eberlein and Behrens (1984). An additional solvent system (pyridine–*n*-butanol–water) that had been used previously (Yih et al. 1968b) was used to move these metabolites from the origin. In this second solvent system, propanil and DCA moved with the solvent front and had identical R_f values (0.97). In the rice extract, propanil, DCA, and two additional metabolites were detected. In S-BYG, all recovered ^{14}C was identified as propanil, but in R-BYG, two additional metabolites were detected with R_f values similar to those of the metabolites found in rice. These were not positively identified by structural analysis, but other researchers studying propanil metabolism in rice using the pyridine–*n*-butanol–water solvent system (Yih et al. 1968b) have identified metabolites with R_f values similar to those of 3,4-dichlorophenyl-glucosylamine (R_f = 0.78) and a 3,4-dichlorophenyl–saccharide conjugate (R_f = 0.60) (Frear and Still 1968; Still 1968; Still and Kuzirian 1967; Yih et al. 1968a, 1968b). The overall degradation scheme of 3,4-dichoroaniline metabolism has been elucidated (Figure 8). It is most likely that the metabolites recovered in our studies are the same conjugates because they were present in both rice and R-BYG, and similar R_f values were obtained using these two solvent systems. These conclusions are supported by the higher level of nonextractable ^{14}C in rice and R-BYG. The extractability of conjugate metabolites decreases in rice as incorporation into plant cell walls, lignin, etc., increases (Still 1968).

These laboratory findings regarding propanil metabolism in R-BYG are also supported by greenhouse results where the resistance mechanism was overcome by the addition of carbaryl. Overall, these data show that the mechanism of resistance in R-BYG is indeed propanil metabolism (Carey et al. 1997; Hoagland et al. 1997).

Mechanisms of herbicide resistance in weeds are generally different from the selectivity mechanisms in the crops on which the herbicides are used (Lebaron and McFarland 1988). For example, triazine resistance is due to an altered herbicide-binding site in the photosynthetic electron transport chain (Radosevich 1977), but crop selectivity is due to metabolic detoxification of the atrazine molecule. A similar pattern is observed in instances of dinitroaniline and sulfonylurea herbicide resistance (Lebaron and McFarland 1988). However, the resistance mechanisms of R-BYG and of R-JR (Leah et al. 1994) are the same as those for the crop (rice). This phenomenon has also been observed in sulfonylurea cross-resistant annual ryegrass (*Lolium rigidum* L.) in Australia (Lebaron and McFarland 1988) and in atrazine resistance in velvetleaf (*Abutilon theophrasti* Medic.) (Anderson and Gronwald 1991).

Several reports have examined propanil tolerance in plants as related to uptake and aryl acylamidase activity. Differences in tolerance to propanil were found in crabgrass [*Digitaria ciliaris* (Retz.) Koeler] and *Echinochloa oryzicola* Vasing seedlings of different ages (Yogo and Ishizuka 1985). Plants became more tolerant with age, and it was suggested that tolerance in crabgrass was due to lower herbicide absorption by shoots rather than to amidase activity. A direct correlation between elevated aryl acylamidase activity and propanil resistance in JR has been demonstrated (Leah et al. 1994). In a subsequent report (Leah et al. 1995), no difference in propanil uptake by R- and S-JR was found, but uptake was reduced as the plants aged. Total activity and specific activity of the amidase activity were higher in resistant vs. susceptible biotypes at all growth stages, but activity also declined with plant age. The authors concluded that lowered uptake confers resistance in older plants. Tolerance of JR and rice to propanil was suggested to be related to lack of retention of propanil on leaf surfaces and to low absorption and translocation (Evbuomwan and Akinyemiju 1995). However, it is not clear whether the biotype of JR used was the wild type or a biotype that had evolved resistance to propanil. JR tolerance to propanil is age dependent (Leah et al. 1995), but for efficacious weed control, injury or mortality of young plants is the primary concern.

It may be possible to use carbaryl or other aryl acylamidase inhibitors as synergists to overcome propanil metabo-

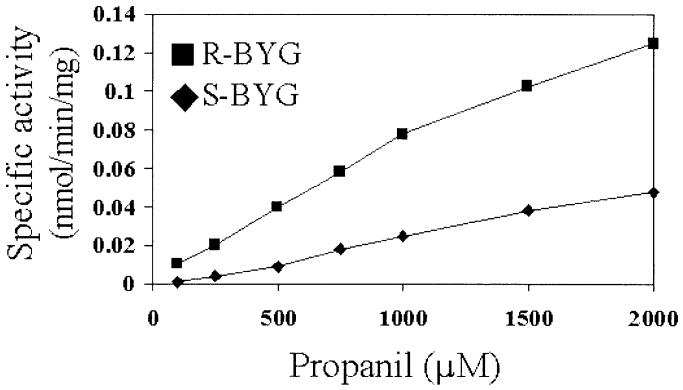

FIGURE 9. Specific activity values of extracted aryl acylamidase from propanil-resistant barnyardgrass (R-BYG, ■) and propanil-susceptible barnyardgrass (S-BYG, ♦) as affected by propanil substrate concentration. (Data from Hirase and Hoagland 2003.)

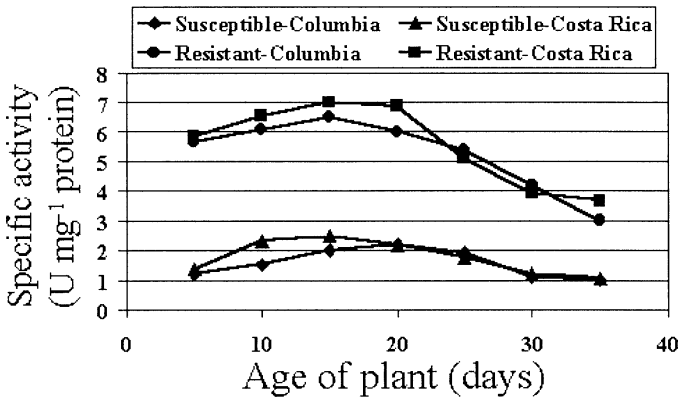

FIGURE 10. Effect of growth stage on specific activity of aryl acylamidase in recently expanded leaves of junglerice. One unit equals one nanomole of dichloroaniline formed per hour. (Adapted and redrawn from Leah et al. 1995.)

lism in R-BYG and control the resistant biotype with propanil. However, because the resistance mechanism of the weed and the selectivity mechanism of the crop are the same, the probability of rice injury would also increase with the use of such a synergist, unless the enzymes in the weed and in rice have diverse kinetic parameters or affinities for substrates and inhibitors or both. Some data using aryl acylamidase inhibitors in vitro indicate slightly greater inhibition by carbamate and organophosphorous insecticides in JR aryl acylamidase than in rice, which may be related to enzyme kinetic parameters (Leah et al. 1994). Attempts were made to synergize or increase control of R-BYG with aryl acylamidase inhibitors and other chemicals using whole-plant screening in the field and greenhouse (Kitt 1995). Other data on propanil interactions with various chemicals is presented in a later section.

Evaluation of Metabolism as a Resistance Mechanism in R-JR

Propanil metabolism in JR (four-leaf stage) in Columbian and Costa Rican biotypes indicated a lower rate of metabolism in the sensitive vs. resistant biotypes (Leah et al. 1995). Results indicated that in addition to DCA, glucosyl-DCA and other unidentified polar metabolites were produced. These data are similar to the metabolite profiles found for rice (Frear and Still 1968) and R-BYG seedlings (Carey et al. 1997).

Although elevated levels of aryl acylamidase activity are responsible for resistance to propanil in one BYG biotype and one JR biotype, no rigorous testing of other propanil-resistant *Echinochloa* spp. has been reported. Because other resistance mechanisms are possible, it cannot be assumed that increased propanil metabolism is the operative mechanism in these other propanil-resistant *Echinochloa* spp. However, the successful use of piperophos or anilofos (recently shown to inhibit aryl acylamidase in vitro [Hirase and Hoagland 2003]) in tank mixtures with propanil to control R-JR in Central America (Valverde et al. 2000) points to a metabolic mechanism.

In Vitro Studies of Aryl Acylamidases from Propanil-Resistant *Echinochloa* spp.

Recently, the aryl acylamidase from R-BYG was isolated and partially characterized (Hirase and Hoagland 2003). Generally, levels of extracted enzyme from the resistant biotype increased linearly over a 5-h assay time course, while activity in the sensitive biotype was two- to threefold lower, and activity tended to decrease at later time points. Specific activity of extracted aryl acylamidase from R-BYG was substantially greater than that of S-BYG (Figure 9). Apparent K_m values were 62 and 3 mM for the enzyme in sensitive and resistant biotypes, respectively. The herbicides anilofos and piperophos also were found to be potent in vitro inhibitors of the enzyme.

Elevated aryl acylamidase was also found in the R-JR biotype (Leah et al. 1995) (Figure 10). Specific activity of this enzyme using propanil as substrate was threefold higher in the R- than in the S-JR biotype and was about 80% of the value found for rice enzyme preparations. Both the total and specific enzyme activity increased with JR plant age up to 15 d (four-leaf stage) and then decreased after 20 d to about half of the maximum at 36 d. Uptake and metabolism studies of propanil in S-and R-JR indicated no significant differences between the biotypes at any growth stage, but uptake was significantly reduced in older plants (Leah et al. 1995). Biochemical analysis of partially purified aryl acylamidases from JR and rice seedlings indicated that these enzymes possessed similar pH optima (pH 7.5) and native molecular masses as estimated by gel filtration (Leah et al. 1997). Kinetic analysis showed that the JR enzyme had a lower affinity for propanil than the rice enzyme. Partially purified aryl acylamidase from rice has an affinity for propanil threefold higher than that of the enzyme in R- and S-JR (Leah et al. 1995). Activity of these enzymes on several substrates showed the same relative order of substrate preference in rice and in S- and R-JR, and the relative rates of activity on each substrate were rice > R-JR > S-JR (Leah et al. 1994) (Table 1). Carbamate and organophosphorus pesticides were inhibitory to enzyme activity in these partially purified rice and JR preparations.

Genetic Considerations of Propanil Resistance in *Echinochloa* Species

Random amplified polymorphic DNA (RAPD) has been used to assess the genetic diversity of BYG in Arkansas and the origin and dispersal of R-BYG (Rutledge et al. 2000). R- and S-BYG biotypes from Arkansas and one S-BYG bio-

TABLE 1. Substrate specificity of aryl acylamidase from crude extracts of rice and propanil-resistant and -susceptible junglerice biotypes.[a]

Substrate	Aryl acylamidase activity		
	Rice (tolerant)	*Echinochloa colona* (resistant)	*E. colona* (susceptible)
	units g^{-1} fresh weight		
Propanil	6,312	5,588	2,672
4-Chloroacetanilide	5,960	5,164	2,592
Acetanilide	5,544	4,944	2,476
4-Methylacetanilide	2,888	1,156	500
4-Chloroacetoacetanilide	1,820	972	456

[a] Adapted from Leah et al. (1994).

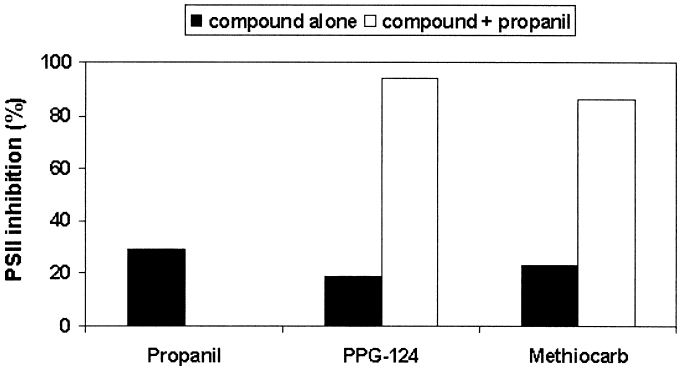

FIGURE 11. Effect of propanil alone (100 μM) and in combination with PPG-124 (50 μM) or methiocarb (50 μM) on photosynthesis in propanil-resistant barnyardgrass leaf disks as measured by chlorophyll fluorescence. (Adapted from Hoagland et al. 1999.)

type from Mississippi were determined to have two distinct genetic clusters, with resistant and susceptible biotypes existing in both clusters. Because of the vast genetic difference between clusters, it was suggested that there are two, rather than one, *Echinochloa* species. Because resistant biotypes from different parts of Arkansas were nearly genetically identical, it was suggested that R-BYG had spread throughout Arkansas by seed dispersal after independent mutation events (Rutledge et al. 2000). On the basis of these findings, these authors concluded that simple control of seed dispersal will not stop the spread of R-BYG. In other genetic analyses, researchers used a RAPD–polymerase chain reaction technique to assess the genetic similarity of *Echinochloa* spp. in southern Europe and found three discrete clusters among five different species (Lopez-Martinez et al. 1999). These analyses, however, did not distinguish between BYG and *E. hispidula* or between *E. oryzicola* and early watergrass on the basis of presence–absence data for 238 loci. Recently, the genetic diversity in *Echinochloa* spp. collected from different countries was examined using amplified fragment length polymorphism and microsatellites (or simple sequence repeats) (Danquah et al. 2002).

Propanil Interactions with Other Chemicals and Alternative Methods to Control Resistant *Echinochloa* Species

Interactions between chemicals (herbicides or others) when tank mixed, applied simultaneously, or in close temporal proximity to a herbicide can alter weed control efficacy. An interaction is the joint action of agrochemicals on plant tissues, and interactions are generally classified as synergistic, additive, or antagonistic (Hatzios and Penner 1985). Such interactions are dependent on the plant species from which the response is measured, and although a synergistic interaction of a chemical with a herbicide is preferred, compounds that produce additive effects also can be useful. This is especially true, for example, when herbicides with different modes of action are tank mixed to effectively control a resistant weed or to prevent resistance development. Possible synergy-causing mechanisms important in weed control are increased herbicide absorption and translocation and decreased herbicide metabolism, which can increase the amount of active herbicide reaching the target site (Duke 1985; Hatzios and Penner 1985).

Various strategies to combat R-BYG and to more fully understand the biochemistry involved in resistance have been investigated. These include interaction of herbicides and other chemicals with propanil (Hoagland et al. 1999; Norsworthy et al. 1999a, 1999b), rotation with other crops, and consequently other herbicides, in rice fields (Kitt 1995), and use of alternative rice herbicides (Baltazar and Smith 1994). Before the development of R-BYG, propanil mixtures with other herbicides for weed control in rice had been evaluated (Smith 1965). Furthermore, the effects of mixtures of propanil and some other rice herbicides on S-BYG control have also been tested (Crawford and Jordan 1995; Jordan 1997; Jordan et al. 1998). Some carbamate and organophosphate insecticides that inhibited aryl acylamidase also synergized propanil phytotoxicity in rice (Bowling and Hudgins 1966; Khodayari et al. 1986; Matsunaka 1968; Smith and Tugwell 1975; Wills and Street 1988). Generally, the carbamate insecticides were more potent enzyme inhibitors than the organophosphorus insecticides (Chang et al. 1971; Frear and Still 1968). Another compound, PPG-124 (*p*-chlorophenyl *N*-methylcarbamate), that lacks insecticidal or herbicidal activity has been commercialized as a herbicide synergist for amide herbicides including propanil (Anonymous 1983). PPG-124 and the insecticide methiocarb gave potent synergistic responses in R-BYG as measured by laboratory bioassay methods, i.e., chlorophyll fluorescence analysis of PSII inhibition (Hoagland et al. 1999) (Figure 11) and reduction of total chlorophyll content (Hoagland et al. 1999) (Figure 12).

As previously mentioned, some carbamate and organophosphate insecticides cause synergistic interactions with propanil (Yih et al. 1968a), a concept also useful in the management of R-BYG. In field studies in Arkansas, interactions between propanil and the herbicides anilofos, pendimethalin, and piperophos and the insecticide carbaryl were synergistic on R-BYG at several rates and resulted in effective control of R-BYG without substantially injuring rice (Hoagland et al. 1999; Norsworthy et al. 1999a). Anilofos plus propanil treatment gave synergistic responses on R-BYG for all treatments containing 0.83 and 3.3 kg ha^{-1} propanil (Norsworthy et al. 1999a) (Table 2). Applications of propanil at 1.65 kg ha^{-1} plus anilofos at 1.0 kg ha^{-1} were also synergistic. Under laboratory conditions using a chlorophyll fluorescence bioassay, anilofos plus propanil treatments were also synergistic (Norsworthy et al. 1999b). Propanil plus carbaryl combinations were injurious to rice

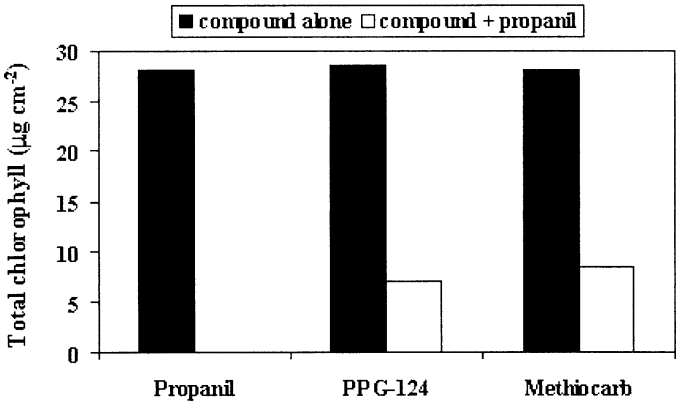

FIGURE 12. Effect of propanil alone (200 μM) and in combination with PPG-124 (100 μM) or methiocarb (100 μM) on total chlorophyll content of propanil-resistant barnyardgrass leaf disks. (Adapted from Hoagland et al. 1999.)

(Norsworthy et al. 1999a), but rice injury as high as 56% at 13 DAT caused by 3.3 kg ha^{-1} propanil plus 0.33 kg ha^{-1} carbaryl had no adverse effects on rice yield (Talbert et al. 1996). Furthermore, the rice herbicides quinclorac and thiobencarb exhibited additive interactions with propanil and also effectively controlled R-BYG.

Unfortunately, it is a very labor-intensive process to field test a vast number of compounds for possible interactions because of the need to evaluate numerous rate combinations. For this reason, chlorophyll fluorescence measurements of R-BYG leaf segments were effectively used to ascertain electron transport inhibition and to assess the synergism and antagonism of propanil with other compounds. In laboratory bioassays, this method was effective in identifying synergists with propanil on R-BYG (Hoagland et al. 1999; Norsworthy et al. 1999b). Also, it was demonstrated that exposure of R-BYG leaf disks to propanil plus an additive and then spectrophotometrically quantifying total chlorophyll was effective to assess compounds for synergy (Hoagland et al. 1999). Thus, it was possible to rapidly appraise synergistic interactions in the laboratory with similar results in the field. Such laboratory screening techniques may lead to other chemical combinations useful for R-BYG control.

Insecticides that inhibit aryl acylamidase activity, such as carbaryl and parathion-methyl, also synergized propanil injury in R-JR seedlings (Caseley et al. 1996). Furthermore, selectivity in rice seedlings and synergy against R-JR was achieved by applying a mixture of propanil with piperophos. Treatment of JR leaves with aryl acylamidase inhibitors such as carbaryl or piperophos combined with propanil significantly overcame the resistance mechanism and increased injury in plants at growth stages where aryl acylamidase activity was maximal (Leah et al. 1995). Inhibitors such as tridiphane blocked 3,4-dichloroaniline peroxidase activity and further metabolism of DCA in intact plants and were found to synergize propanil phytotoxicity against R-JR. 3,4-Dichloroaniline has some degree of phytotoxicity in JR (Caseley et al. 1996). Mixtures of both inhibitors (aryl acylamidase and peroxidase) were the most potent against the resistant biotype (Figure 13). Interactions of propanil in two- and three-way combinations with piperophos and the herbicide tridiphane showed that the three-way mixture was the most effective treatment in reducing R-JR fresh weight.

Several strategies to control propanil-resistant *Echinochloa* spp. have been entertained or tested. These include the use of alternative herbicides with modes of action different from that of propanil, propanil mixed with other herbicides, propanil mixed with aryl acylamidase inhibitors, and biocontrol with bioherbicidal microorganisms.

TABLE 2. Determination of synergistic, antagonistic, or additive effects from observed and expected control of two- to three-leaf propanil-resistant barnyardgrass (R-BYG) from propanil plus anilofos combinations 7 d after treatment.[a]

Propanil	Anilofos	R-BYG control		Interaction
		Observed	Expected	
kg ha^{-1}		%		
0.83	0.11	58	35	Synergistic
	0.33	77	41	Synergistic
	1.0	74	43	Synergistic
	3.0	84	58	Synergistic
1.65	0.11	70	59	Additive
	0.33	82	63	Additive
	1.0	89	64	Synergistic
	3.0	90	73	Additive
3.3	0.11	89	63	Synergistic
	0.33	94	66	Synergistic
	1.0	95	68	Synergistic
	3.0	98	76	Synergistic
6.6	0.11	95	91	Additive
	0.33	98	92	Additive
	1.0	98	92	Additive
	3.0	100	94	Additive
LSD (0.05)		21		

[a] Data from Norsworthy et al. (1999a).

Some herbicides such as pendimethalin have only low efficacy on BYG when applied postemergence, but when combined with propanil the mixture gives excellent control of S-BYG and R-BYG (Norsworthy et al. 1999a; Smith and Baltazar 1993; Walton and Holmdal 1992). Pendimethalin applied after propanil improved control of R-JR (Riches et al. 1997). Interactions among propanil; the herbicides anilofos, pendimethalin, piperophos, quinclorac, and thiobencarb; and the insecticide carbaryl were evaluated under field conditions to discover possible synergistic or additive interactions useful to control R-BYG without injuring rice. Propanil and each additive were evaluated at four rates, for a total of 16 rate combinations for each additive. Anilofos, carbaryl, piperophos, or pendimethalin in combination with propanil produced synergistic effects on R-BYG based on

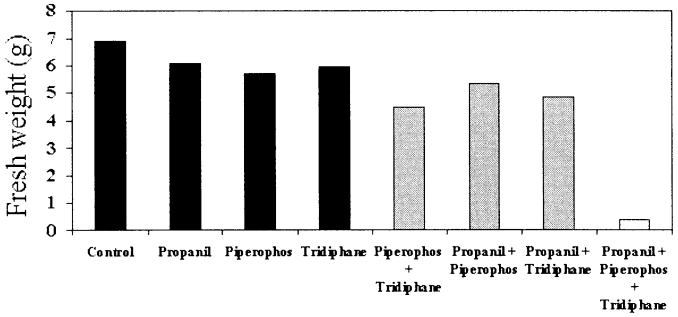

FIGURE 13. Effect of 0.75 kg ha^{-1} propanil, 0.3 kg ha^{-1} piperophos, and 0.3 kg ha^{-1} tridiphane alone and in two- and three-way mixtures on the fresh weight of propanil-resistant junglerice treated at the four-leaf growth stage. (Adapted and redrawn from Caseley et al. 1996.)

weed control ratings. For each additive tested, at least one rate combination with propanil controlled R-BYG > 80% with minimal rice injury (< 20%). Piperophos and quinclorac mixed with propanil provided control of R-JR and increased rice yields (Valverde 1996; Valverde et al. 1997). Control of initial R-JR coupled with crop–weed management regimes can serve as the basis for integrated control of herbicide-resistant JR (Valverde et al. 2001).

Concluding Remarks and Future Research Areas

Biotypes of two *Echinochloa* species have become resistant to propanil and some other herbicides, and these biotypes have been found in various parts of the world. The existence of these resistant biotypes is highly correlated with the use of propanil for extended time periods. Although numerous reports have been published on propanil-resistant biotypes of JR and BYG, only two specific propanil-resistant biotypes have received rigorous study. In those two cases, the mechanism of resistance has been shown to be elevated propanil metabolism by aryl acylamidase. On the other hand, because of the relatively large number of propanil-resistant *Echinochloa* accessions reported, it is quite possible that nonmetabolic resistance mechanisms exist, similar to the situation found in ryegrass biotypes in Australia (Powles et al. 1997). The diverse taxonomic nature of *Echinochloa* spp. and their propanil-resistant biotypes and other biotypes resistant to other herbicides strongly suggests that more research is needed in the genetic analysis of this plant group. As pointed out, the age of test plants plays a major role in the resistance of both these biotypes to propanil because both absorption and metabolism of this herbicide vary greatly with growth stage. Genetic analysis and proteomics may elucidate mechanisms of gene and enzyme regulation in the wild-type and herbicide-resistant biotypes. To combat this resistance problem, various compounds have been tested in the laboratory and in the field to find effective synergists with propanil. Additionally, alternative herbicides with modes of action different from those of propanil have been used to control these weeds. The development of resistance to a particular herbicide can be reduced if herbicides with different modes of action are used alternately to control targeted weeds (Powles et al. 1997). Use of such strategies could help prevent the spread of these resistant biotypes to areas not currently infested. Lastly, biochemical research is needed to discover other potent synergists and to isolate, purify, and characterize the aryl acylamidase in the resistant biotypes with respect to kinetic parameters in order to determine the nature of the interaction of inhibitors and assess the intricacies of their binding sites.

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